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All Mouse Ventral Spinal Cord Patterning by Hedgehog Is Gli Dependent and Involves an Activator Function of Gli3

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Summary

An important question is how the gradient of Hedgehog is interpreted by cells at the level of the Gli transcription factors. The full range of Gli activity and its dependence on Hh have not been determined, although the Gli2 activator and Gli3 repressor have been implicated. Using the spinal cord as a model system, we demonstrate that Gli3 can transduce Hedgehog signaling as an activator. All expression of the Hh target gene *Gli1* is dependent on both *Gli2* and *Gli3*. Unlike Gli2, however, Gli3 requires endogenous *Gli1* for induction of floor plate and V3 interneurons. Strikingly, embryos lacking all *Gli* function develop motor neurons and three ventral interneuron subtypes, similar to embryos lacking Hh signaling and Gli3. Therefore, in the spinal cord all Hh signaling is Gli dependent. Furthermore, a combination of Gli2 and Gli3 is required to regulate motor neuron development and spatial patterning of ventral spinal cord progenitors.

Introduction

The Hedgehog (Hh) family of secreted proteins control patterning, growth, and morphogenesis of many tissues in both vertebrates and invertebrates (for review, see Ingham and McMahon, 2001). In flies, the pathway is relatively simple since there is only one Hh protein and the cellular response to Hh signaling is mediated by the single transcription factor Cubitus interruptus (Ci). Hh regulates whether the bipartite Ci protein acts as an activator or repressor. In the presence of Hh signaling, processing of Ci into an N-terminal repressor is inhibited and full-length Ci is turned into a labile activator (Méthot and Basler, 1999; Ohlmeyer and Kalderon, 1998). Furthermore, the activator function of Ci requires higher levels of Hh than inhibition of processing. Thus, specific concentrations of Hh produce different responses due to the relative amount of active Ci repressor and activator (Aza-Blanc and Kornberg, 1999). In *hh* mutants, the Ci repressor is upregulated, whereas in *ci* mutants, some cells take on the fate of cells receiving intermediate levels of Hh where the Ci activator and repressor counteract each other. In mammals, the Hh pathway is more complicated, in part because there are three Gli transcription factors that mediate Hh signaling. An important question is how the Gli proteins function in response to different concentrations of Hh. One prediction based on Ci is that each of the Gli proteins will function as an activator at high levels of Hh, repressor in the absence of Hh, and as a graded combination of activator and repressor at intermediate levels.

The spinal cord has been used as a model to study components of the Hh pathway since it is a relatively simple structure. Six distinct classes of neurons are generated in the spinal cord, with floor plate (FP) forming in the ventral midline and V3 interneurons, motor neurons (MN), V2, V1, and V0 interneurons forming from ventral to dorsal (reviewed in Jessell, 2000). *Smo*, an obligate component of Hh signaling, is required for development of all ventral spinal cord cell types, and Sonic Hh (Shh) is the primary Hh in this process (Pierani et al., 1999; Wijgerde et al., 2002). In addition, Shh is expressed in a ventral-to-dorsal decreasing gradient in the spinal cord (Gritli-Linde et al., 2001) and is sufficient in vitro to induce at least five of the ventral cell types in a concentration-dependent manner (reviewed in Jessell, 2000). In the present study, we address how the Gli proteins respond to this gradient and produce different cell types.

Of greatest difference from Ci, mouse Gli1 contains only an activator domain and does not undergo proteolytic processing (Dai et al., 1999; Park et al., 2000). Consistent with this, in gain-of-function assays in embryos, mouse Gli1 can induce the expression of Hh targets in the absence of Hh (Hynes et al., 1997; Lee et al., 1997; Park et al., 2000). Mice homozygous for *Gli1* mutations, however, do not have developmental defects or a decrease in Hh signaling, unless one copy of *Gli2* is also removed (Bai et al., 2002; Park et al., 2000). Like Ci, mouse Gli2 and Gli3 contain repressor and activator domains on their N- and C termini, respectively (Dai et al., 1999; Sasaki et al., 1999), but numerous studies have shown that Gli2 is the primary Hh-dependent activator.

In mouse, *Gli2* is required in the spinal cord for development of FP and most V3 interneurons, cell types that require high-level Shh signaling (Ding et al., 1998; Matise et al., 1998). Since *Gli1;Gli2* double mutants lack all V3 interneurons and $Gli1^{-/-};Gli2^{+/-}$ mutants have a reduction in FP and V3 cells (Matise et al., 1998; Bai et al., 2002; Park et al., 2000), Gli1 enhances the activator function of Gli2 in inducing the most ventral cell types. Furthermore, in fly imaginal discs or cell culture, Gli2 activates target genes in response to Hh (Aza-Blanc et al., 2000; Sasaki et al., 1999). Also, unlike Ci, frog Gli2 appears to undergo Hh-independent proteolytic processing into a repressor, at least when expressed in fly imaginal discs (Aza-Blanc et al., 2000). However, if a mouse Gli2 repressor is formed, then it is not actually required during normal mouse development, since *Gli1* expressed in place of *Gli2* can rescue the spinal cord and all other *Gli2* mutant defects (Bai and Joyner, 2001).

Similar to Ci, Gli3 can be proteolytically processed into a C-terminal truncated repressor in embryos, and processing is inhibited by Hh signaling (Litingtung et al.,

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2002; Wang et al., 2000). While a full-length Gli3 protein can be detected in embryos, an activator function for Gli3 has not been demonstrated during normal development. Only in cell culture assays or *Ptc* mutants has Gli3 been shown to function as a weak activator (Dai et al., 1999; Motoyama et al., 2003; Shin et al., 1999), although deletion of the repressor region of Gli3 produces a potent activator (Sasaki et al., 1999). Loss of *Gli3* results in many phenotypes reminiscent of ectopic activation of the Shh pathways (Hui and Joyner, 1993). In the spinal cord, *Gli3* mutants have a dorsal expansion of V0-V2 interneurons (Persson et al., 2002). Significantly, this defect is rescued by expression of an N-terminal repressor form of Gli3 from the endogenous *Gli3* locus (Persson et al., 2002). Whether the Gli proteins normally play a role in MN development is not clear, as MNs form in *Gli1;Gli2* or *Gli3* mutants. The repressor function of Gli3 is also revealed by removing *Gli3* in *Shh* or *Smo* mutant embryos, as this rescues the loss of all but FP and V3 interneurons in the spinal cord, as well as digit formation in the limbs (Litingtung and Chiang, 2000; Litingtung et al., 2002; te Welscher et al., 2002; Wijgerde et al., 2002).

Electroporation of a Gli3 repressor into chick embryos blocks formation of all ventral cell types, and this was taken to suggest that Gli activity is required for development of all ventral spinal cord cell types (Persson et al., 2002). Consistent with this, *Gli2*$^{-/-}$*;Gli3*$^{-/-}$ mutants lacking one copy of *Ptc* do not form motor neurons at E9.5, although more dorsal cell types were not analyzed (Motoyama et al., 2003). In contrast, the formation of MNs and V0-V2 interneurons in *Shh;Gli3* or *Smo;Gli3* double mutants and analysis of a Shh responsive gene, *CoupTFII*, indicate that a Gli-independent pathway could regulate some ventral spinal cord development (Krishnan et al., 1997; Litingtung and Chiang, 2000; Wijgerde et al., 2002). However, it is possible *Gli1* and *Gli2* function independent of Hh in *Smo;Gli3* mutants. Finally, while some phenotypes of *Gli2*$^{-/-}$*;Gli3*$^{-/-}$ embryos are more severe than in either single mutant (reviewed in Ingham and McMahon, 2001), it is not clear whether the overlapping functions reflect an activator role for Gli3 and/or a repressor role for Gli2.

We took a number of in vivo approaches to determine whether Gli3 normally functions as an activator, and whether ventral spinal cord cell types can develop in the absence of all Gli function. First, we show that there is a reduction in Hh positive signaling in *Gli3* mutants and a complete loss of expression of the Hh target *Gli1* in *Gli2;Gli3* double mutants. Thus, *Gli3* contributes in a positive manner to Hh signaling, and transcription of *Gli1* is absolutely dependent on both *Gli2* and *Gli3*. In complimentary studies, we directly tested the in vivo activator function of Gli3 in production of floor plate and V3 interneurons by expressing *Gli3* in place of *Gli2*. We show that Gli3 indeed has weak activator function in patterning the ventral spinal cord since it can induce some floor plate and V3 interneurons. However, unlike Gli2, the activator function of Gli3 is dependent on *Gli1* transcription. Last, by analyzing mice lacking all *Gli* function at E10.5, we show that MNs and more dorsal interneurons can form in the ventral spinal cord in the absence of all Gli function. Unlike *Gli1;Gli2* or *Gli3* mutants, however, the three ventral spinal cord cell types form in abnormal positions. There also is a striking increase in proliferation of ventral progenitors, including motor neuron progenitors. Thus, a combination of Gli2 and Gli3 functions regulate the spatial patterning and proliferative roles of Hh signaling in the spinal cord. These studies, taken together with previous mutant studies, allow us to define the roles of each *Gli* gene in multiple aspects of early ventral spinal cord development, and to conclude that all Hh signaling is dependent on Gli function.

Results

A Positive Contribution of Gli3 to Hh Signaling

As one approach to determine whether Gli3 normally functions as a Hh-dependent activator, we examined expression of *Gli1* in *Gli* mutants. We recently demonstrated that transcription of *Gli1* in the spinal cord is absolutely dependent on *Shh* (Bai et al., 2002), unlike another Hh target, *Ptc*, which is expressed in *Smo* mutants (Wijgerde et al., 2002). We used a *lacZ* knockin allele of *Gli1* in which β-galactosidase (β-gal) activity recapitulates *Gli1* expression, and therefore can be used as a sensitive readout of positive signaling by Hh (Bai et al., 2002). In wild-type (wt) embryos at E10.5, *Gli1-lacZ* is expressed strongly in the ventral CNS and posterior mesenchyme of the limb bud (Figures 1A and 1D). Consistent with the idea that the activator function of Gli2 is a major component of positive Shh signaling, *Gli1-lacZ* expression was severely reduced in the ventral CNS and limb buds of *Gli2* mutant embryos (Figures 1B and 1E). However, β-gal activity was not completely abolished in *Gli2* mutants, raising the possibility that *Gli3* is responsible for some *Gli1* expression.

We next examined Hh signaling in *Gli3* mutant embryos. At E10.5, the overall level of *Gli1-lacZ* expression was lower in the CNS of *Gli3* mutant embryos than in wt embryos (Figures 1C and 1F). In particular, β-gal activity in the posterior limb bud was greatly reduced, as previously reported (Litingtung et al., 2002). To compare Hh signaling in wt and *Gli3* mutant embryos in more detail, we examined the expression of *Gli1-lacZ* in E9.5 spinal cord sections. In wt sections, β-gal activity can be detected in the ventral spinal cord after 3 hr of staining. In contrast, only very weak β-gal activity could be detected in *Gli3* mutant ventral spinal cord sections (Figures 1G and 1H). The staining became more obvious in both genotypes after overnight incubation, but wt spinal cord sections showed a broader expression domain than *Gli3* mutants. Our finding that *Gli1-lacZ* is reduced in *Gli3* mutants suggests a positive contribution of Gli3 to Hh signaling, rather than Gli3 only being a repressor of Hh targets that must be removed for gene activation.

Both Gli2 and Gli3 Are Required to Activate *Gli1*

Our finding that loss of either *Gli2* or *Gli3* leads to differential downregulation of Hh signaling raised the possibility that there are overlapping functions in the activator functions of Gli2 and Gli3. To test this possibility, embryos homozygous for both *Gli2* and *Gli3* were generated and analyzed for *Gli1-lacZ* expression. At E8.5, E9.5, and E10.5, the expression of *Gli1-lacZ* in *Gli2;Gli3*

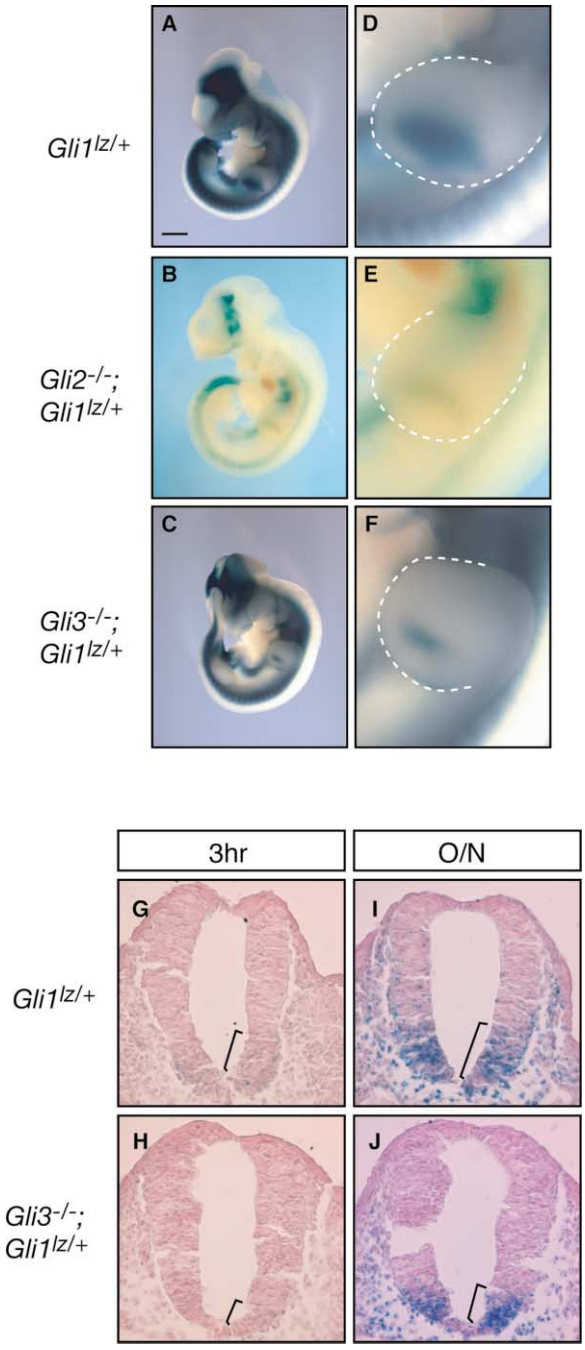


Figure 1. Reduced *Gli1-lacZ* Expression in *Gli3* Mutant Embryos

(A–F) Whole-mount X-gal staining of *Gli1-lacZ* at E10.5. (D–F) Higher power images of forelimbs (outlined by white dots) of embryos shown in (A)–(C).

(G–J) X-gal staining of *Gli1-lacZ* in cervical spinal cord sections at E9.5. (G) and (H) were stained for 3 hr, (I) and (J) overnight. Brackets indicate the extent of staining.

Scale bar: (A–C) 1 mm, (D–F) 0.22 mm, (G–J) 50 μm.

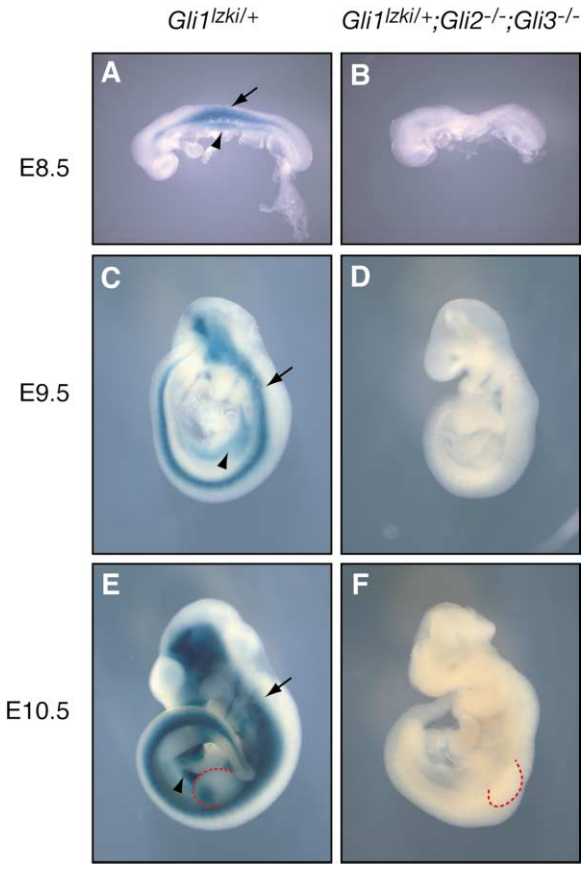


Figure 2. Both *Gli2* and *Gli3* Are Required for Expression of *Gli1*

(A, C, and E) In wt embryos, *Gli1-lacZ* can be detected in the CNS (arrow), gut (arrowhead), and limb bud (outlined by red dots) from E8.5 to E10.5.

(B, D, and F) In *Gli2;Gli3* embryos, *Gli1-lacZ* expression is completely absent in all tissues.

double mutant embryos was completely abolished throughout the embryo (Figure 2). In contrast, in *Shh* mutant embryos *Gli1-lacZ* is expressed only near the gut in response to Ihh (Bai et al., 2002; unpublished data); thus Gli2 and Gli3 mediate all positive Hh signaling in the mouse embryo. Furthermore, *Gli2;Gli3* double mutant embryos are equivalent to *Gli1;Gli2;Gli3* triple mutant embryos, since *Gli1* is not expressed.

Expression of Gli3 in Place of Gli2 Using a Knockin Gene Targeting Approach

Although it is possible that the Gli3 repressor normally represses a *Gli1* repressor, the downregulation of Hh signaling in *Gli3* mutants is most likely due to loss of a Gli3 activator since the *Gli1* gene contains DNA binding sites for Gli3 (Dai et al., 1999). In order to directly test whether Gli3 can function as an activator in mediating normal Shh signaling, we replaced *Gli2* with *Gli3*. A human *GLI3* cDNA linked to the mouse *Gli2* 3′UTR and three copies of an SV40 polyadenylation signal was inserted into the first coding exon of the endogenous *Gli2* locus (Figure 3). Our previous studies showed that insertion of a cDNA into the *Gli2* locus using an identical gene targeting strategy results in expression of the exogenous cDNA precisely in the *Gli2* expression domain, and at the same time inactivates the endogenous *Gli2*

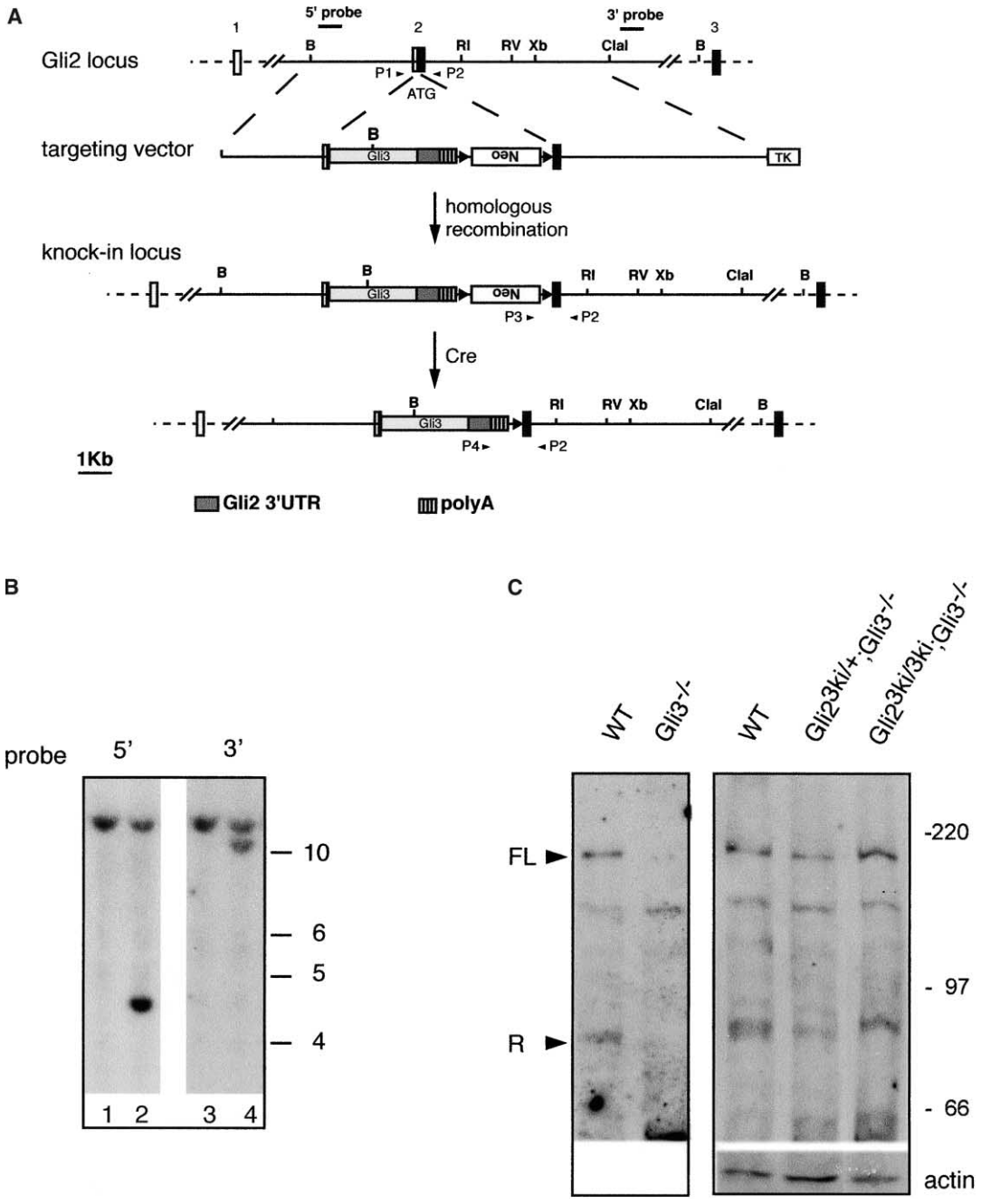


Figure 3. Strategy to Express *GLI3* in Place of *Gli2*

(A) *Gli2* genomic locus, targeting construct, and knockin allele (with and without *neo*). White boxes represent *Gli2* untranslated exons, and black boxes represent translated exons. P1-P4 represent PCR genotyping primers. Arrowheads indicate loxP sites. Transcription of *neo* is in the opposite direction of *Gli3*.

(B) Southern blot analysis of ES cell DNA digested with BamHI. Lanes 1 and 3 are from wt ES cells, lanes 2 and 4 from targeted cells. The size in kb is shown on the right.

(C) Western blot analysis of GLI3 protein expressed from the *Gli2* locus. Gli3 can be detected as full-length (FL) and truncated (R) forms in wild-type (WT) but not $Gli3^{-/-}$ embryos. When *GLI3* is expressed in place of *Gli2*, FL and R forms are detected. Note that there is slightly more full-length GLI3 in the $Gli2^{3ki/3ki};Gli3^{-/-}$ embryos than in $Gli2^{3ki/+};Gli3^{-/-}$ embryos. For the trunk sample, the region between the forelimbs and hindlimbs (with heart and gut removed) were used.

gene (Bai and Joyner, 2001). Furthermore, insertion of a *Gli2* cDNA rescues the *Gli2* mutant defects.

The initial *Gli2* knockin allele that was generated contains a floxed neomycin resistance gene (*neo*) that can be removed by breeding with *TK-Cre* mice. Removal of *neo* in such knockin alleles was previously shown to enhance the expression of the inserted cDNA (Bai and Joyner, 2001). Two independently targeted lines of mice

with and without *neo* were generated and found to have the same phenotypes. Heterozygous mice in which one allele of *Gli2* is replaced by *GLI3* ($Gli2^{n3ki/+}$ and $Gli2^{3ki/+}$, where 3ki represents *GLI3* knock-in and n indicates presence of the *neo* cassette) appeared normal.

In order to assess GLI3 protein produced from the *Gli2* knockin allele, we bred the $Gli2^{3ki}$ allele onto a $Gli3^{xt}$ homozygous mutant background and analyzed Gli3 protein by Western blotting. In wt embryos, both full-length (Gli3-FL) and the processed repressor form of Gli3 (Gli3-R) are detected in mouse limbs (Wang et al., 2000). In $Gli3^{xt}$ homozygous mutants ($Gli3^{-/-}$), neither Gli3 protein was detected (Figure 3C). When GLI3 was expressed from the *Gli2* locus in the absence of endogenous *Gli3*, the two GLI3 protein forms were detected in E10.5 trunk or limb samples (Figure 3C and data not shown). Furthermore, the GLI3-FL/GLI3-R ratio remained roughly the same in wt (0.96), $Gli2^{3ki/+}$;$Gli3^{-/-}$ (1.00), or $Gli2^{3ki/3ki}$;$Gli3^{-/-}$ embryos (0.99). As expected, more GLI3 protein was detected (35% more) when two copies of *GLI3* were expressed in place of *Gli2* compared to one copy (Figure 3C). We then examined whether the inserted *GLI3* cDNA can functionally rescue the limb phenotypes seen in $Gli3^{-/+}$ mice. Indeed, in 8 out of 11 $Gli2^{3ki/+}$; $Gli3^{+/-}$ mice examined, the polydactyly phenotype was fully rescued and the remaining 3 had a near complete rescue compared with their $Gli3^{+/-}$ littermates (n = 8 mice) (Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/1/103/DC1]).

Gli3 Can Function as an Activator to Rescue Some Gli2 Mutant Spinal Cord Defects

If Gli3 can function as an activator, then the defects associated with loss of *Gli2* should be rescued in $Gli2^{3ki/3ki}$ embryos, as when *Gli1* replaces *Gli2* (Bai and Joyner, 2001). We therefore determined whether FP and V3 interneurons form by examining E10.5 spinal cord sections. We first analyzed $Gli2^{3ki/3ki}$ embryos that express the highest level of *Gli3* in place of *Gli2*. Indeed, some Shh-expressing FP cells were detected at all anterior/posterior (A/P) levels of the $Gli2^{3ki/3ki}$ spinal cord (Figure 4A and data not shown). The extent of the Shh domain, however, was smaller than in wt embryos. In addition, a near-normal number of Nkx2-2-expressing V3 interneurons were found at all levels of $Gli2^{3ki/3ki}$ embryos, although they were situated in the ventral midline of the spinal cord. Furthermore, in $Gli2^{3ki/3ki}$ embryos Isl1/2-expressing MNs were found in a similar lateral position of the spinal cord as wt embryos throughout the spinal cord. The phenotype of $Gli2^{3ki/3ki}$ embryos is similar to that in $Gli1^{-/-}$;$Gli2^{-/+}$ embryos (see Figure 7A). Our analysis of $Gli2^{3ki/3ki}$ embryos demonstrates that at levels similar to normal *Gli2* expression, *Gli3* can partially rescue the *Gli2* mutant spinal cord phenotype throughout the spinal cord.

We next examined embryos expressing a lower level of *Gli3* in place of *Gli2* ($Gli2^{n3ki/n3ki}$) and found that some Shh-expressing FP cells formed in the cervical and FL regions of the spinal cord, but not in the trunk and HL regions (data not shown). In addition, more Nkx2-2-expressing V3 interneurons were generated in the cervical and FL regions than in *Gli2* mutant embryos, and Isl1/2-expressing MNs were not found in the ventral midline but were situated in a more lateral position. In the trunk and HL regions of the $Gli2^{n3ki/n3ki}$ spinal cord, however, V3 interneurons and MNs appeared in a similar pattern to that in *Gli2* mutant embryos (data not shown).

In embryos with the lowest level of *Gli3* expressed in place of *Gli2* ($Gli2^{n3ki/-}$, where "−" represents a null allele with *lacZ* replacing *Gli2*), no Shh-expressing FP cells were detected throughout the A/P axis of the spinal cord (Figure 4A). Overall, the phenotype of $Gli2^{n3ki/-}$ embryos was similar to that of *Gli2* mutant embryos, demonstrating that a low level of *Gli3* is not sufficient to substitute for the activator function of Gli2. This is in contrast to *Gli1* (or *Gli2*), which can completely rescue all *Gli2* mutant defects when expressed at a low level in $Gli2^{n1ki/-}$ (or $Gli2^{n2ki/-}$) embryos (Bai and Joyner, 2001).

To further analyze the spinal cord rescue of *Gli2* mutants by *Gli3*, the *Gli1-lacZ* allele was crossed onto $Gli2^{3ki/3ki}$ knockin background to provide an additional readout of positive Shh signaling. As expected, the level of *Gli1-lacZ* detected in the spinal cord of E9.5 $Gli2^{3ki/3ki}$ embryos was elevated compared to *Gli2* mutants (Figure 4B). The expression of *Gli1-lacZ* in knockin embryos is consistent with the partial rescue of the floor plate by the Gli3 activator in $Gli3^{3ki/3ki}$ knockin embryos.

The Activator Function of GLI3 in $Gli2^{3ki/3ki}$ Embryos Requires Gli1 and Endogenous Gli3

It is possible that the activator function of Gli3 by itself is sufficient to induce floor plate cells and a large number of V3 interneurons, similar to *Gli1* when it is expressed in place of *Gli2* and in the absence of endogenous *Gli1* (Bai et al., 2002). Alternatively, Gli3 could induce the expression of endogenous *Gli1*, which then rescues the floor plate defect. To discriminate between these two possibilities, *Gli1* function was removed from embryos homozygous for the $Gli2^{3ki}$ allele. Strikingly, in such $Gli2^{3ki/3ki}$;$Gli1^{lz/lz}$ embryos, Shh-expressing FP cells were not detected throughout the A/P axes of the spinal cord (Figure 4A). In addition, only a very small number of Nkx2-2-expressing V3 interneurons were detected in the ventral midline of a few $Gli2^{3ki/3ki}$;$Gli1^{lz/lz}$ spinal cord sections (data not shown). Isl1/2-expressing MNs were found to occupy the ventral midline of the spinal cord, and thus the overall phenotype was similar to *Gli1;Gli2* double mutants (see Figure 7A). This result demonstrates that when *Gli3* is expressed in place of *Gli2*, it induces transcription of endogenous *Gli1*, and the Gli1 activator then induces the Shh target genes necessary for floor plate formation and production of a normal number of V3 interneurons.

We next addressed whether the endogenous *Gli3* gene contributes to the partial rescue of FP and V3 interneurons in $Gli2^{3ki/3ki}$ embryos by breeding the mutants onto a $Gli3^{xt}$ mutant background. Strikingly, the ventral spinal cord of a $Gli2^{3ki/3ki}$;$Gli3^{-/-}$ embryo (n = 2) appeared similar to *Gli2* null mutant embryos. Shh-expressing FP cells were not detected, a small number of Nkx2-2 V3 interneurons occupied the ventral midline, and Isl1/2 was expressed across the ventral midline (data not shown). This result is consistent with the poor rescue seen when *Gli3* was expressed at lower levels in $Gli2^{n3ki/n3ki}$ embryos, and lack of rescue in $Gli2^{n3ki/-}$ embryos. Moreover, the experiment shows that Gli3 protein

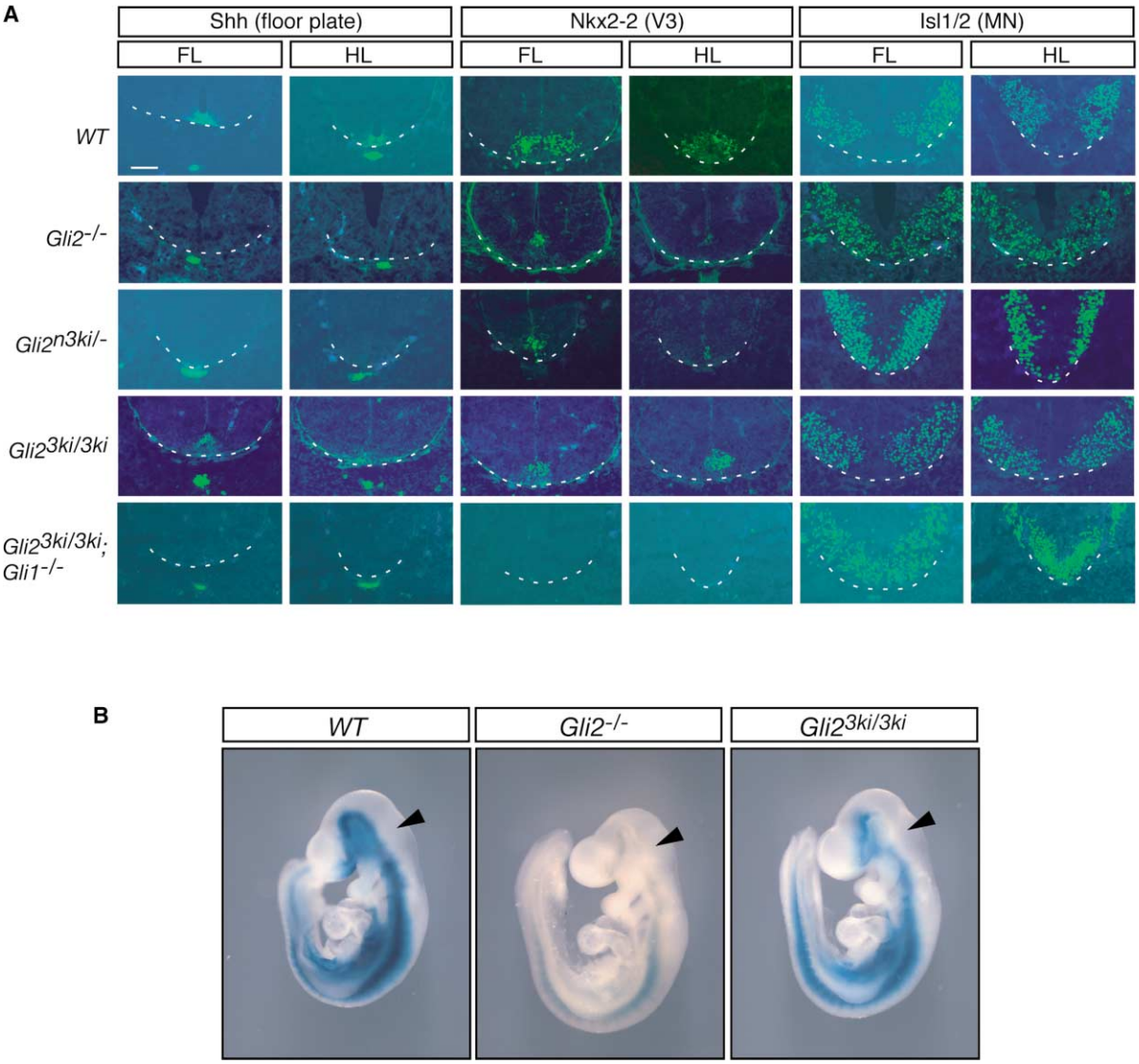


Figure 4. Activator Function of Gli3 Can Partially Induce Floor Plate Cells and V3 Interneurons

(A) Forelimb (FL) and hindlimb (HL) sections from the mutants shown and stained with antibodies to the proteins indicated.
(B) X-gal staining of *Gli1-lacZ* showing a substantial rescue of *Gli1-lacZ* expression in *Gli2*$^{3ki/3ki}$ embryos compared to *Gli2*$^{-/-}$ embryos except in the brain (arrowhead) and posterior regions.
Scale bar: (A) 50 μm, (B) 0.4 mm.

expressed from the endogenous *Gli3* locus contributes to the rescue in *Gli2*$^{3ki/3ki}$ embryos. Thus, the endogenous Gli3 protein can contribute activator function in the ventral spinal cord to induce FP and V3 interneurons.

Motor Neurons and V0-2 Interneurons Develop in the Absence of Gli Function

To test whether the presence of ventral spinal cord cell types, including MNs, in *Shh;Gli3* and *Smo;Gli3* mutants is due to a Hh-independent function of *Gli2* and *Gli1,* we examined whether ventral cell types can form in the absence of *Gli* function. We first examined the spinal cords of E10.5 *Gli2;Gli3* double mutants that lack all Gli function (Figure 2) for expression of MN markers. Significantly, at cervical, forelimb, and trunk, but not hindlimb levels, Isl1/2-expressing MNs were detected in all double mutant embryos. However, the number of MNs was reduced in *Gli2;Gli3* double mutants compared to wt embryos, and the number and distribution varied somewhat between mutants. At E9.5, a few MN were present in wt, but not *Gli2;Gli3* double mutants (Supplemental Figure S2). The reduction of MN at E10.5 was further illustrated by counting the number of MNs in four double mutants (138 ± 91), compared to two wt embryos (267 ± 25). An earlier marker for differentiating MNs, HB9/MNR2, also detected a reduced number of cells occupying the ventral midline of the spinal cord (Figure 5H).

In agreement with previous studies showing that Gli activators are required in the ventral-most spinal cord, Shh-expressing floor plate cells and Nkx2-2-expressing

V3 interneurons were not found in *Gli2;Gli3* double mutant embryos (Figures 5F and 5G). Of interest, expression of Pax6, which normally has a ventral limit at the level of the MN progenitors, was found to extend to the ventral-most region of the spinal cord of *Gli2;Gli3* mutants, whereas the ventral border of the dorsal progenitor marker Pax7 did not shift (Figures 5I and 5J). Since $Pax6^{+}/Pax7^{-}$ cells normally mark the MN and V0-2 interneuron progenitors, the Pax6 and Pax7 expression patterns indicated V0-V2 cells could be formed in the absence of Gli function.

We therefore examined markers for specific postmitotic ventral neuron cell types in wt and mutants lacking all Gli function. In wt embryos at E10.5, V2 interneurons express Chx10, V1 interneurons express En1, and V0 interneurons express Evx1/2. In addition, these three cell types and MNs occupy stereotypical locations in the ventral spinal cord (Figures 5K–5N). In *Gli2;Gli3* double mutant embryos, even though MN and V2 interneurons were detected at the forelimb level, these two cell types intermingle with each other (Figure 5O). V1 (En1) interneurons also were found scattered throughout the ventral spinal cord, and although the V0 interneurons were generally found in more dorsal positions, they spread more ventral than normal (Figure 5Q). At hindlimb levels, fewer V2, V1, and V0 interneurons were present (Figures 5P and 5R).

We next addressed whether the progenitors for the ventral cell types were generated in the correct positions in the ventricular zones, or whether *Gli* mutant progenitors also lose their positional information and occupy incorrect positions in the ventricular zone. In the ventral spinal cord, Dbx1 marks V0 progenitors; Nkx6.1 marks V2, MN, and V3 progenitors; Olig2 marks MN progenitors; and Nkx2.2 marks V3 progenitors. Similar to the differentiated neurons, V0 progenitors remained dorsal but were expanded slightly ventral in *Gli2;Gi3* embryos (Figure 5W), and progenitors expressing Nkx6.1 were expanded ventrally to the midline (Figure 5X). Strikingly, MN progenitors expressing Olig2 were detected throughout the ventral spinal cord, similar to Nkx6.1 expressing cells (Figure 5Y). Furthermore, in contrast to differentiated MNs, there were more MN progenitors in the double mutants (166 ± 19, $n = 2$ embryos) than in wt embryos (60 ± 2, $n = 2$ embryos). At E12.5, the Olig2 domain remained expanded in the one embryo that survived to this stage (Supplemental Figure S2). At E9.5, Olig2 MN progenitors were not detected at the cervical level in *Gli2;Gli3* mutants ($n = 2$), but were present in reduced numbers at the forelimb level compared to wt (Supplemental Figure S2). As described above, Nkx2-2 expressing progenitors for V3 interneurons could not be detected in *Gli2;Gli3* mutant spinal cords (Figure 5Z). These results demonstrate that Gli function is not required for the generation of progenitors of four ventral cell types (V0-V2 and MN), but instead is required for their correct positioning and number, and possibly for differentiation of MNs.

To determine whether one allele of *Gli2* or *Gli3* is sufficient for motor neuron development, we analyzed Olig2 and Isl1/2-expression in $Gli2^{-/-};Gli3^{-/+}$ and $Gli2^{-/+};Gli3^{-/-}$ mutant embryos. Interestingly, in both mutants, the MN progenitor domain was greatly expanded dorsal, whereas MN appeared to differentiate normally. Thus, one allele of *Gli2* or *Gli3* is sufficient to promote MN differentiation, but not to suppress the expansion of the progenitor domain (Supplemental Figure S3).

Abnormal Proliferation of Ventral Progenitors in the Absence of Gli Function

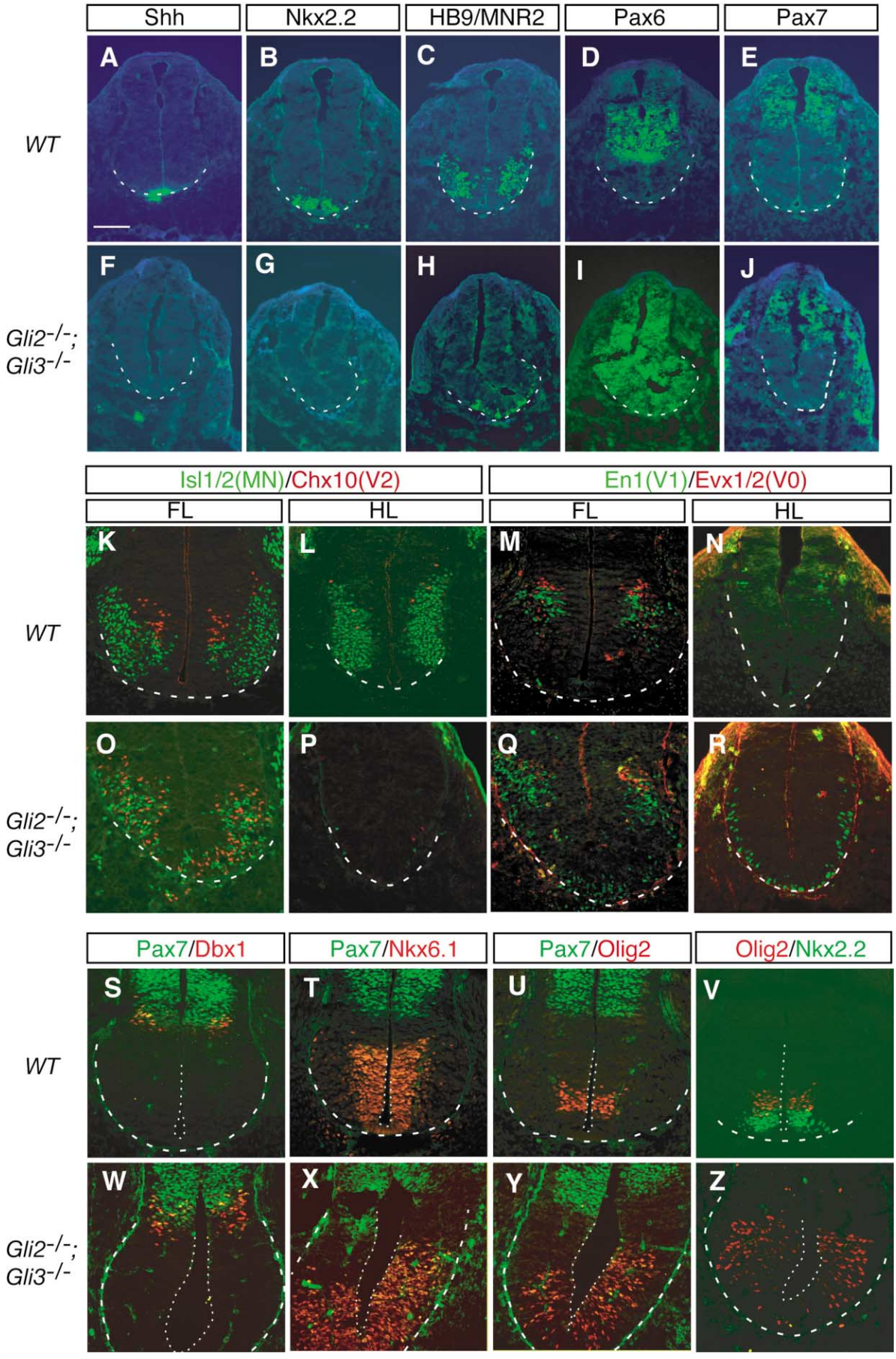
In addition to the phenotype of intermingled ventral progenitors and neurons, another obvious phenotype in mutant embryos lacking all *Gli* function was an abnormal wavy morphology reflecting an expansion of the ventral spinal cord in all double mutant embryos examined (Figures 5 and 6). This phenotype was not seen in $Gli2^{-/-};Gli3^{-/+}$ or $Gli2^{-/+};Gli3^{-/-}$ mutant embryos (Supplemental Figure S3). To examine whether overproliferation accompanies the phenotype in *Gli2;Gli3* mutant ventral spinal cords, we analyzed BrdU incorporation. At E10.5 in wt embryos, BrdU was found to be incorporated primarily in the intermediate spinal cord with a decreasing gradient ventrally (Figure 6). In *Gli2* or *Gli3* single mutants, the profile of BrdU incorporation in the spinal cord was quite similar to that in wt embryos. In contrast, in embryos lacking all Gli function, BrdU incorporation was greatly increased in the ventral spinal cord, including the ventral midline. Furthermore, unlike *Gli2* or *Gli3* single mutants, the majority of the spinal cord appeared to be occupied by ventricular zone progenitors, as revealed by nuclear staining (Hoechst).

One possible reason for the increase in incorporation of BrdU in the spinal cord of mutants lacking all *Gli* function is a disruption of the cell cycle. To address this, we examined Cyclin D1 expression in *Gli2;Gli3* mutant embryos, since Cyclin D1 is a G1 Cyclin that has been shown to be a target of Shh signaling and downregulated in *Gli2* mutant hair follicles (Kenney and Rowitch, 2000; Mill et al., 2003). In wt embryos at E10.5, Cyclin D1 was detected primarily in progenitors in the intermediate region of the spinal cord (Figure 6). In *Gli2* mutants, Cyclin D1 expression was similar to or slightly reduced compared to wt embryos. In *Gli3* mutants, Cyclin D1 expression appeared slightly upregulated, although the overall spatial pattern was similar to wt embryos. In contrast and consistent with the BrdU staining, Cyclin D1 expression in *Gli2;Gli3* mutant embryos was upregulated throughout the ventral spinal cord. In addition, although in both $Gli2^{-/-}$ and *Gli2;Gli3* double mutant embryos floor plate and V3 progenitors are missing, only in *Gli2;Gli3* mutant embryos was BrdU incorporation and Cyclin D1 expression detected in the ventral midline. These results demonstrate that loss of Gli function results in disruption of cell cycle control and overproliferation of ventral spinal cord progenitors.

Discussion

Mouse Gli3, Along with Gli2, Induces the Hh Target Gene *Gli1*

By using a sensitive marker for Shh positive function, we demonstrate that loss of *Gli3* results in reduced Hh signaling throughout the CNS at E9.5 and E10.5, as well as in the limb buds at E10.5. We further demonstrated an overlap in activator functions between Gli2 and Gli3 using two in vivo approaches. First, in *Gli2;Gli3* double homozygous mutant embryos, all *Gli1-lacZ* expression


Shh
Nkx2.2
HB9/MNR2
Pax6
Pax7
A
B
C
D
E
WT
F
G
H
I
J
Gli2-/-;
Gli3-/-
Isl1/2(MN)/Chx10(V2)
En1(V1)/Evx1/2(V0)
FL
HL
FL
HL
K
L
M
N
WT
O
P
Q
R
Gli2-/-;
Gli3-/-
Pax7/Dbx1
Pax7/Nkx6.1
Pax7/Olig2
Olig2/Nkx2.2
S
T
U
V
WT
W
X
Y
Z
Gli2-/-;
Gli3-/-

is absent, whereas it is only reduced in either single mutant (Figures 1 and 2). Given that *Gli1-lacZ* is a readout of the positive action of Hh signaling, this demonstrates a loss of the activator functions of both Gli2 and Gli3 in these embryos. We then directly tested the activator function of Gli3 during normal Hh signaling in the spinal cord by expressing human *GLI3* in place of the endogenous *Gli2* gene. Our study demonstrates that Gli3 protein expressed from the endogenous *Gli3* allele, along with GLI3 protein from the $Gli2^{3ki/3ki}$ knockin allele, function as activators to induce the expression of *Gli1* and development of some FP cells and many V3 interneurons (Figure 4).

Although an internal deletion of the N terminus turns both Gli2 and Gli3 into strong activators of the Hh target *Hnf3*β (Sasaki et al., 1999), our studies show that the inherent activator function of Gli3 is not as potent as Gli2. First, *Gli3* cannot fully rescue FP and V3 interneuron induction in the spinal cord of *Gli2* mutants. Second, unlike Gli2 (or Gli1), Gli3 cannot induce FP and V3 interneurons in the absence of the endogenous *Gli1* gene when expressed like *Gli2* (Figure 7A). Furthermore, *Gli3* cannot completely rescue other *Gli2* mutant phenotypes, such as the lung defects, and $Gli2^{3ki/3ki}$ pups live for only 2 days after birth (data not shown). Consistent with our results, Gli3 appears to only weakly contribute to positive Hh signaling in *Ptc* mutants in which the pathway is highly activated. Removal of *Gli2*, but not *Gli3*, rescues the expanded FP and V3 interneuron phenotype in *Ptc* mutants, whereas removal of both *Gli2* and *Gli3* leads to a suppression of MN development, at least at E9.5 (Bai et al., 2002; Motoyama et al., 2003).

The fact that Gli3 cannot fully rescue many *Gli2* defects demonstrates that Gli2 and Gli3 proteins have distinct intrinsic biochemical properties. One possibility is that Gli2 and Gli3 recognize different subsets of target genes with different affinities. Indeed, Gli1 and Gli3 have different affinities for *Gli* DNA binding sites (Nakashima et al., 2002). Alternatively, or in addition, Gli3 but not Gli2 forms a potent repressor and thus there may be some Gli3 repressor made in the ventral spinal cord of $Gli2^{3ki/3ki}$ embryos that competes with any activator produced. Consistent with this, our Western blot analysis showed that a substantial amount of the Gli3 expressed from the *Gli2* locus is in the repressor form. Thus, changing the expression domain of *Gli3* to that of *Gli2* is not sufficient to remove all Gli3 repressor.

Generation of Most Ventral Progenitors Does Not Require Gli Function

A central argument for the morphogen model of Shh activity is the fact that particular neurons are induced at specific Shh concentrations in spinal cord explants (reviewed in Jessell, 2000). How the cells read this gradient at the molecular level has not been clear. A surprising finding was that when *Gli3* is removed from *Smo* mutant embryos MNs and V0-2 interneurons form, albeit in abnormal positions (Wijgerde et al., 2002). Thus an essential requirement for Hh signaling in these four ventral cell types must be to inhibit formation of the Gli3 repressor. Since Hh signaling is lost in these mutants, it is possible that formation of Gli2 or Gli3 activators is also required in these cells. In addition, because *Gli2* is expressed in the double mutant background, it was not known whether *Gli2* has an Hh-independent function in generating the remaining ventral cell types. Furthermore, it was not known whether *Gli2* and *Gli3* play redundant roles in MN and V0-V2 development, since although a recent study concluded that *Gli2* and *Gli3* are required for MN development, the analysis was only done at E9.5 and V0-V2 interneurons were not examined (Motoyama et al., 2003). We have resolved these questions by demonstrating that Gli proteins are not required for generation of many MNs and V0-V2 interneurons at E10.5, but instead they are required to regulate normal MN differentiation, as well as for correct spatial patterning of ventral cell types (Figure 5).

The experiments presented here, taken together with other studies of mouse *Gli* mutants, allow us to define the activator and repressor functions of each Gli protein in transducing the Hh gradient in the mouse ventral spinal cord (summarized in Figure 7B). First, high and low levels of Shh downregulate formation of the Gli3 repressor to create a permissive environment for ventral spinal cord cell types. Second, at the highest levels of Hh, Gli2 and Gli3 activators are formed and they induce *Gli1* expression. Third, the Gli2 activator, and to a much lesser extent Gli1, generate V3 interneurons and FP in a concentration-dependent manner. Fourth, at moderate levels of Hh it is likely that a combination of Gli2 and Gli3 activators are made and these ensure normal proliferation and differentiation of MN progenitors. Fifth, at low levels of Hh the Gli3 repressor is sufficiently repressed to allow a normal number and distribution of V2-V0 interneurons to develop. Some level of Gli3 repressor is, however, required for normal development of V0-V2 interneurons (Persson et al., 2002), whereas Gli1 and Gli2 activators are not required. Gli2 and Gli3 are also required for the distinct spatial organization of MNs and V0/V1 progenitors.

Our studies show that in the absence of Gli function, the ventral spinal cord is not converted into one cell type that normally forms in response to an intermediate level of Hh where the activators and repressor are equally balanced. Furthermore, there must be transcription factors other than the Gli proteins that induce differentiation of V0-V2 interneurons and some MNs. Retinoic

Figure 5. Ventral Spinal Cord Defects in Embryos Lacking All *Gli* Function

Sections from *Gli2;Gli3* and wt embryos stained with antibodies to the proteins indicated.

(A–J) Floor plate (Shh) and V3 interneurons (Nkx2.2) are not present, MNs (HB9/MNR2) are reduced, Pax6 is expanded ventral, and Pax7 is normal.

(K–R) MN, V2, V1, and V0 interneurons can be detected in *Gli2;Gli3* mutant embryos, but are intermixed in the ventral spinal cord. Note: fewer postmitotic neurons were detected in the hindlimb (HL) than forelimb (FL) region of *Gli2;Gli3* mutant embryos than in wt embryos.

(S–Z) Sections stained for progenitors of MN (Olig2), V3 (Nkx2.2), V1 (Dbx1), and V2 + MN + V3 (Nkx6.1). Progenitors for MN, V1, and V2 are present but intermixed.

Scale bar: (A–J) 50 μm, (K–Z) 94 μm.

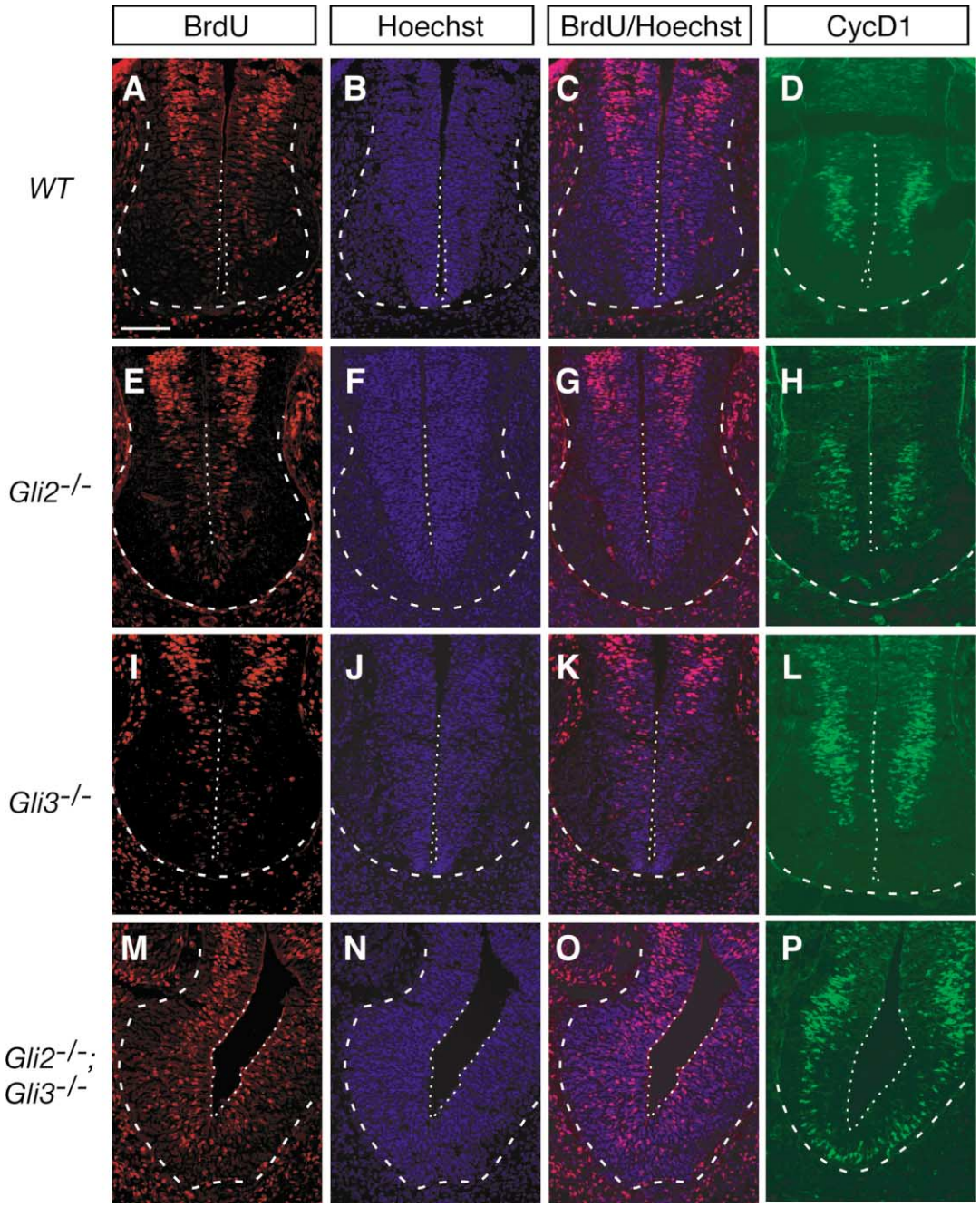


Figure 6. Overproliferation in Embryos Lacking *Gli* Function Is Accompanied by Upregulation of Cyclin D1

In spinal cord sections, proliferating progenitor cells in the ventricular zones are labeled with BrdU and Cyclin D1 by antibody staining. Nuclei are marked with Hoeschst. The pattern of BrdU incorporation remains largely unchanged in *Gli2*$^{-/-}$ or *Gli3*$^{-/-}$ embryos (E–G, J–K) compared to wt (A–C), but is expanded in the ventral spinal cord in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ embryos (M–O). Cyclin D1 is slightly reduced in *Gli2*$^{-/-}$ embryos (H), slightly increased in *Gli3*$^{-/-}$ embryos (L), and upregulated throughout the ventral spinal cord in *Gli2;Gli3* mutants (P) compared to wt (D). The contour of the spinal cord and spinal canal is outlined by dotted lines. Scale bar: 50 μm.

acid is likely one of these molecules (Pierani et al., 1999). Interestingly, if Hh produces a gradient of Gli3 repressor, then the gradient is not required to induce the different V0-V2 cell types, since in the absence of Gli3 V0 and V1 interneurons are not converted into V2 interneurons (or MNs). There are, however, redundant functions of Gli2 and Gli3 in regulating the spatial organization of MNs and V0/V1 interneurons and their progenitors, since in *Gli2; Gli3* mutants, unlike *Gli3* mutants, the three cell types are intermixed (Persson et al., 2002; Figure 5 and unpublished data). In contrast to the spinal cord where a combination of Gli1/2 activators and Gli3 repressor are needed, in the limb only the Gli3 repressor plays the primary role in development, since *Gli1;Gli2* mutants

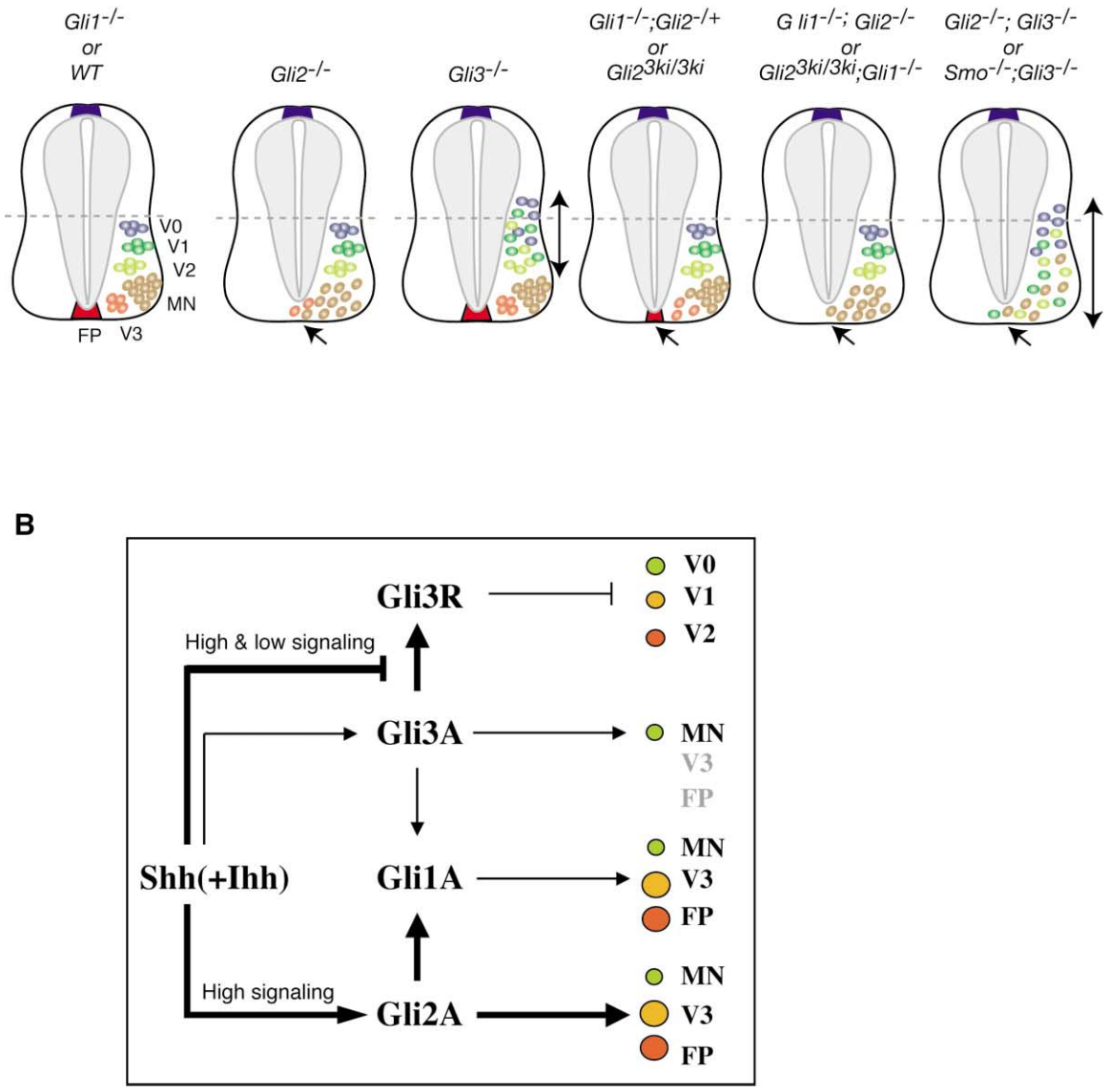


Figure 7. The Role of Each Gli Protein in Mouse

(A) Summary of the mouse *Gli* mutant spinal cord phenotypes at E10.5. The expanded ventral spinal cord in *Gli2;Gli3* and *Smo;Gli3* mutants is not shown. (B) Specific contribution of Gli activators to the generation of floor plate, V3 interneurons, and MNs. Thickness of lines and size of circles indicate relative contributions. See text for details.

have normal A/P patterning (Park et al., 2000) and *Shh;Gli3* mutant limbs have a phenotype similar to *Gli3* mutants (Litingtung et al., 2002; te Welscher et al., 2002).

Redundant Functions of Gli2 and Gli3 in Motor Neuron Development and Spatial Patterning of Progenitors

A requirement for Gli genes in MN development has not previously been clear, since an apparently normal number of MNs form in *Gli1;Gli2* or *Gli3* mutants (Ding et al., 1998; Matise et al., 1998; Persson et al., 2002). Our present study has uncovered specific functions for Gli proteins in MN development. In embryos lacking all Gli function, there is a reduction in differentiated MNs but an increase in their progenitors (Figures 5 and 6). The increase in progenitors cannot be the only cause for the reduced differentiation, since in $Gli2^{-/-};Gli3^{-/+}$ or $Gli2^{-/+};Gli3^{-/-}$ mutant embryos the MN progenitors are increased but differentiation proceeds normally (Supplemental Figure S3). An additional unexpected finding

is that the ventral spinal cord is expanded in *Gli2;Gli3* mutants (Figures 5 and 6). Interestingly, both these phenotypes, as well as disorganization of MNs, V1, and V2 interneurons, also are seen in *Smo;Gli3* mutants (Wijgerde et al., 2002; data not shown). In addition, the expression domain of CyclinD1 and incorporation of BrdU are increased in the ventral spinal cord of *Gli2;Gli3* mutants. This raises the possibility that Gli2 and Gli3 normally downregulate CyclinD1 to allow proper differentiation of postmitotic MNs.

Since *Smo;Gli3* mutants have the same phenotype as *Gli2;Gli3* mutants, and no Gli2 activator can be formed in either mutant combination, the MN defect is likely due to loss of redundant activator functions of Gli2 and Gli3. In this case, an inhibitor of CyclinD1 might be a target of the Gli activators. Since the Gli3 repressor inhibits proliferation in some tissues, an alternative is that the Gli3 repressor inhibits proliferation and CyclinD1 and therefore the Gli2 activator and Gli3 repressor have redundant, but different, roles in regulating MN development. An interesting extension of this would be that a graded balance between the Gli3 repressor and Gli activators normally produces the distinct progenitor domains in the ventral spinal cord.

All Early Hh Signaling in the Spinal Cord Requires Gli Function

If all Hh signaling in mice is dependent on the three *Gli* genes and only Gli3 acts as a repressor, then *Smo;Gli3* double mutant embryos should have the same phenotype as mice lacking all Gli function. Indeed, we show that this is the case in the spinal cord (Figure 7A). The similar phenotype of *Smo;Gli3* and *Gli2;Gli3* mutants at E10.5 provides definitive proof that all early Hh signaling is dependent on Gli function. Furthermore, the required functions of the Glis in the spinal cord must be Hh regulated, with the possible exception of the dorsal d4-6 subtypes that are reduced in *Gli3* mutants (Persson et al., 2002). In this regard, it is curious to note that *Gli2* and *Gli3* are expressed extensively in the dorsal spinal cord; however, in *Gli2;Gli3* mutant embryos there is no obvious additional phenotype not seen in *Gli3* mutants.

Evolution of the Ci/Gli Proteins

During evolution, three mouse *ci/Gli* genes have taken the place of one ancestral gene. In addition, the mechanisms for regulating the vertebrate *Gli* genes by Hh signaling has become much more complex and seems to be different even between fish and mouse (Karlstrom et al., 2003). One major change is that transcription of mouse *Gli1* is regulated by Hh signaling (reviewed in Ingham and McMahon, 2001) and dependent on *Gli2* and *Gli3* (Figures 1 and 2). Hh may also regulate Gli function at the translational level by inhibiting GLI1 protein production through sequences in the 3′UTR (Jan et al., 1997). Inhibition of processing of Gli3 most closely resembles that of Ci (Wang et al., 2000), and our studies show that production of a Gli activator is conserved to the greatest extent in mouse Gli2. In addition, while a level of redundancy has been conserved in the activator functions of the three mouse Gli proteins, only Gli3 has a repressor function. During evolution, therefore, the various functions of an ancestral protein have to a large extent been distributed between the three Gli proteins in mouse, and new mechanisms for regulating Gli function have been developed.

Experimental Procedures

Generation of Gli3 Knockin Mice and Mouse Breeding

A human *Gli3* cDNA (Ruppert et al., 1990) corresponding to the coding region was tagged with a FLAG epitope (Sigma) at the 5′ end by PCR and the mouse *Gli2* 3′UTR and three tandem repeats of an SV40 polyA signal were added to the 3′ end. The resulting construct was inserted into the first translated exon of *Gli2* by homologous recombination, essentially as described (Bai and Joyner, 2001). ES cells were screened by Southern blot analysis using BamHI digestion and eight targeted cell lines identified (1/13). The 5′ probe identifies a band of 4.5 kb in targeted ES cells and a >13 kb band in wt cells. The 3′ probe identifies a band of 11 kb in targeted ES cells and a >13 kb band in wt cells. Two lines of mice were generated from independent ES cell clones and bred to *TK-Cre* mice to remove the selection marker *neo*. Noon of the day of detection of a vaginal plug was considered E0.5. The genotyping of $Gli1^{lz,\ Gli2zfd}$, and $Gli3^{xt}$ (Jackson Laboratory) and knockin mice was as described (Bai and Joyner, 2001; Maynard et al., 2002). All but $Gli3^{xt/+}$ mice (C57) were maintained on a Swiss Webster background.

Immunohistochemistry, X-Gal Staining, BrdU Labeling, and Western Blotting

Embryos were fixed in 4% paraformaldehyde for 20 min at 4°C and equilibrated in 30% sucrose overnight before being embedded in OCT. X-gal staining and antibody staining were performed as described (Bai and Joyner, 2001). Antibodies used in this study have been described (Pierani et al., 1999; Takebayashi et al., 2000), except for anti-Cyclin D1 (Oncogene) and anti-BrdU antibody (Becton-Dickinson). For BrdU labeling, pregnant mice were injected intraperitoneally with BrdU (Sigma) at 100 μg per gram body weight 1 hr prior to dissection. To count the number of neurons, two sections at the forelimb level from each embryo were examined, and two to four embryos of the same genotype were used. The average and standard deviation for each genotype were then calculated.

Western blot analysis was done essentially as described and blotted against rabbit anti-Gli3 antibody (Wang et al., 2000). The membrane was then blotted with anti-actin antibody (Santa Cruz) to control for loading. NIH image was used to digitize and normalize (with actin) the band intensity on scanned images.

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